Drug Repositioning: Auranofin as a Prospective Antimicrobial Agent for the Treatment of Severe Staphylococcal Infections.

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Abstract. Auranofin, (AF), a gold(I) complex in clinical use for the therapy of rheumatoid arthritis, is reported here to produce remarkable bactericidal effects in vitro against Staphylococcus sp. Noticeably, a similar antimicrobial action and potency are also noticed toward a few MRSA strains but not toward E. Coli. The time and concentration dependencies of the antimicrobial actions of AF have been characterized through recording time kill curves, and a concentration dependent profile highlighted. Overall, the present results point out that auranofin might be quickly and successfully repurposed for the treatment of severe bacterial infections due to resistant Staphylococci.

Keywords: Auranofin • Antimicrobial drugs • Gold compounds • Drug repositioning • Staphyloccocal Infections

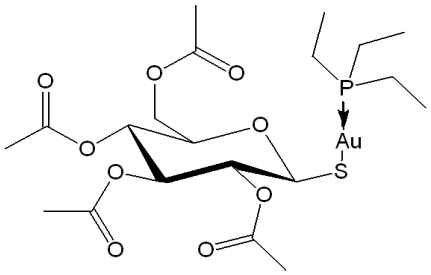

Figure 1. Chemical Structure of Auranofin

Introduction

Drug discovery and development is nowadays a very expensive, time-consuming, and risky process as witnessed by the limited number of new drugs approved every year. Attrition rates are extremely high, even during the late phases of clinical evaluation, with a huge financial impact. To speed up drug discovery and development and reduce failure rates and the associated costs, drug repositioning or repurposing may be considered as a highly effective and promising strategy (Chong et al. 2007, Aroson 2007).

Drug repositioning involves the investigation of drugs that were already approved for the treatment of other diseases and/or whose mechanisms or targets are known. Various methods including new screening platforms and advanced *in silico* and bioinformatic approaches may be exploited for the identification of the best candidates for drug repositioning. A few successful stories of drug repositioning are well documented in the recent literature such as those of finasteride, thalidomide, sildenafil and metformin (Cavalla 2013).

Auranofin, AF (**I**), is a mixed ligand gold compound in clinical use since 1985 for the treatment of severe rheumatoid arthritis (**Ridaura**) (Shaw 1999). It consists of a gold(I) center linearly coordinated to a triethylphosphine and a thiosugar ligand as shown in figure 1.

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The presence of the strong phosphine ligand confers favourable pharmacological properties to this gold(I) center while the thiosugar ligand is a somewhat weaker ligand and may be released more easily. Following the release of the thiosugar, a coordination position is made available for gold(I) binding to biomolecules (Zou et al. 2000).

The development of AF as an antiarthritic drug was inspired by the traditional use of various gold compounds in the treatment of this disease dating back to the 1930s'. In comparison to other established antiarthritic gold compounds, AF has the big advantage of being administered orally. Yet, in spite of numerous investigations carried out so far, the actual mechanisms through which AF produces its favourable effects in RA are still unclear and largely unknown. There is just some limited evidence that AF can modulate the immune response and affect inflammation's mediators (Bondenson 1997, Messori et al 2004, Han et al. 2008).

Remarkably, auranofin combines the conspicuous affinity (after activation) of the gold(I) center for various "soft" donors (in particular S and N donors from proteins' side chains), with a moderate and largely acceptable systemic toxicity. This paves the way to its repositioning for new and different therapeutic uses. While the drug repositioning strategy has been extensively applied to several organic drugs, very limited examples still concern the case of inorganic drugs. Nevertheless, in recent years, a number of new attractive pharmaceutical actions were disclosed for AF including promising anticancer, antiviral and antiprotozoan properties;. in particular AF seems to be very effective for the treatment of of *Schistosoma* and also of *Plasmodium falciparum* infections (Shaw 1999, Kuntz 2007, Sanella et al. 2008). The state of art on the new medical uses of AF is described in detail in a comprehensive review article (Madeira et al. 2012).

Specifically, we wondered whether auranofin might possess effective antibacterial properties toward microorganisms that are difficult to treat with the available drugs and antibiotics. This is now a very urgent and unmet medical need as the number of innovative clinically approved antibiotics has dramatically decreased during the last two decades while an increasing number of multi resistant and highly dangerous bacteria (the so called *superbugs*) has appeared. Quite surprisingly, no systematic study exists in the literature on the antibacterial uses of AF; just some fragmentary observations are available (Novelli et al. 1999).

However, very recently, AF has been reported to be highly effective toward *Clostridium difficile* (Jackson-Rosario Self 2010), *Treponema denticola* (Jackson-Rosario Self 2009), and

metronidazole-resistant *Giardia lamblia* (Tejman-Yarden et al. 2013) .

The above arguments led us to explore in more depth the antimicrobial properties of auranofin toward a few representative bacterial strains.

Materials and methods

Strains, antibiotics, and media

The strains used for the experiments were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* USA 300, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* ATCC 35984 (biofilm producer) and 5 recent clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). Fresh dilutions of auranofin were prepared daily.
The in vitro experiments were performed in Mueller Hinton broth and Mueller Hinton agar (Oxoid, UK).

MIC determination

Minimal inhibitory concentrations (MIC) were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and using a broth dilution method. Briefly, two-fold broth serial dilutions were performed at inoculum sizes of $1x10^6$ CFU/ml obtained from a 18-24h incubation at 35°C. Incubation of test tubes, containing a final volume of 1 ml, was done at 35°C for 18h-24h. The MIC was defined as the lowest concentration of antimicrobial agent that inhibits the development of visible growth in the tubes (Clinical Laboratory Standards Institute 2004).

Time-kill curves

The killing activity of auranofin (**I**) and of its analogue (**II**) over time was evaluated in liquid medium (Mueller–Hinton Broth) at 37°C while shaking. The activity was examined against *S. aureus* ATCC 25923 at a final concentration of between 10^6 colony forming units (CFU)/mL and 10^7 CFU/mL in either the presence or absence of auranofin (**I**) or derivative (II) at concentrations of 1/4xMIC, MIC, 2xMIC, 4xMIC, 16xMIC and 64xMIC. Bacterial growth was evaluated at time zero (before the addition of the two complexes) and at 1h, 2h, 4h, 6h and 24h after addition of treatment agents, both in the control and antibiotic samples, using the CFU/mL count method. At each hour, 0.1mL of the sample removed from the bottles were diluted two-fold with normal saline (0.9% NaCl) and spread on Mueller-Hinton agar plates using L-shaped rod and incubated for 24h at 37°C. Colony count of bacteria between 30–300 CFU/mL for each plate was determined to obtain time-kill curves (Clinical Laboratory Standards Institute 2004).

Results and discussion

AF was tested against two reference strains, namely *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 that are representative of Gram negative and Gram positive bacteria, respectively. For comparison purposes parallel experiments were carried out on $AuClPEt_3$ (**II**) an auranofin analogue where the thiosugar ligand is replaced by a chloride ligand (Shaw 1999).

The *in vitro* antimicrobial properties of both auranofin and its analogue were assessed by determination of the minimal inhibitory concentration (MIC) and by time-kill analysis. The high MIC values observed with both gold compounds in the case of *E. coli* ATCC 25922 strain (>8mg/l) point out that both compounds are practically ineffective against *Enterobacteriaceae*. In contrast, both compounds are characterized by a remarkable *in vitro* activity with low MIC values (ranging from 0.125 to 0.5 mg/l) against the *S. aureus* ATCC 25923 strain (Table 1).

	Auranofin (mg/l)	**$AuClP(Et)_3$ (mg/l)**
E. coli ATCC 25922	> 8	> 8
***S. aureus* ATCC 25923**	0.25 - 0.5	0.125 – 0.25
***S. aureus* USA 300**	0.25	0.125
***S. aureus* MRSA 1**	0.25	0.125
***S. aureus* MRSA 2**	0.25	0.125
***S. aureus* MRSA 3**	0.25	0.125
***S. aureus* MRSA 5**	0.125 – 0.25	0.125
***S. aureus* MRSA 6**	0.25	0.125
S. epidermidis ATCC 12228	0.25	0.125
S. epidermidis ATCC 35984 (biofilm producer)	0.25	0.125

Tab 1. MIC values for the two gold complexes Auranofin and $AuClP(Et)_3$ against different bacteria strains (range from 4 experiments).

These observations prompted us to analyse in more detail the *in vitro* activity of **I** and **II** against different reference strains of the *Staphylococcus* genus including *S. epidermidis* and methycillin resistant *S. aureus* (MRSA) and also against recent clinical isolates of MRSA; indeed, this latter pathogen, causing extensive morbidity and mortality is considered worldwide as a very difficult one to treat and eradicate(Karampela et al. 2012). Interestingly, both gold compounds turned out to be very active *in vitro* against all tested strains, including all clinical isolates independently of being methycillin sensitive or resistant and biofilm producer or not. Typically, the resulting MIC values ranged from 0.125 to 0.5 mg/l and from 0.125 to 0.25mg/l, for compound **I** or **II**, respectively, with no significant differences on their *in vitro* antimicrobial potency (Table 1).

Time kill curves offer reliable and detailed information on the mode through which antimicrobial agents cause bacteria's death. The results obtained with the time–kill analysis in the case of **I** and **II** are shown in fig 2 and 3. Notably, such a method may give specific insight on the pharmaco-dynamic properties of the different drugs regarding either their concentration- or time-dependent activity (Craig 1998).

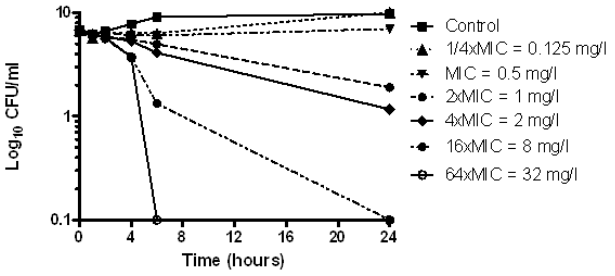

Figure 2. Killing curves with Auranofin against *S. aureus* ATCC 25923 (mean of two experiments)

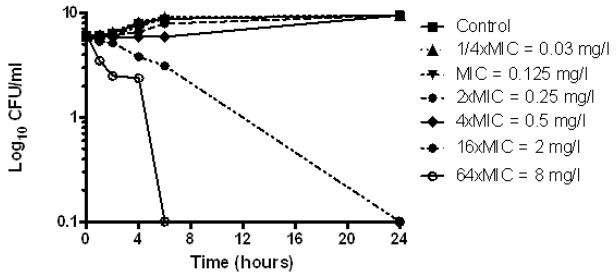

Figure 3. Killing curves with derivative (**II**) against *S. aureus* ATCC 25923 (mean of two experiments).

Analysis of the obtained killing curves indicates that the antibacterial activities of both auranofin and its analogue **II** on the *S. aureus* ATCC 25923 strain (with a MIC of 0.5 mg/L and 0.125 for **I** and **II** respectively) are very pronounced. Auranofin showed inhibition of bacterial growth for 12 h at MIC and for 24 h at 2×MIC, whereas no re-growth was observed even after 24 h at 16 and 64×MIC (fig. 2). Compound **II** showed a somewhat lower antibacterial profile with inhibition of bacterial growth for 8 h at 4xMIC, though no regrowth was observed after 24 h at 16 and 64×MIC (fig. 3). Auranofin demonstrated a concentration-dependent bactericidal activity with sterilization at 6–12 h at concentrations >4×MIC, with a reduction in the bacterial count of 3 log compared to the control in the first 6 h (fig. 2).

Owing to this data, complexes **I** and **II** may be classified as strong antimicrobial agents versus *S. aureus* and *S. epidermidis* strains. Their antimicrobial properties are very similar both in terms of potency and of the spectrum of sensitive strains. As complexes **I** and **II** only differ in the nature of the more labile ligand (the tetracetylthioglucose ligand in **I** and chloride in **II**) it is straightforward to assume that such ligand just plays an ancillary role in the overall antimicrobial activity while the triethylphosphine ligand constitutes an important part of the pharmacophore. The detailed mode of bacteria growth inhibition caused by these gold compounds will be the subject of further mechanistic studies; on the basis of existing literature, we can just state that there are two plausible mechanisms that warrant specific investigation, namely interference with selenium metabolism and inhibition of thioredoxin reductase and related enzymes (Jackson-Rosario Self 2009).

Overall, in view of the here reported *in vitro* results, it is evident that the clinically established antiarthritic drug auranofin demonstrates a high bactericidal activity against *Staphylococcus* sp; a similar antimicrobial potency is manifested versus the MRSA strains. Based on a preliminary set of experiments, the activity of auranofin appears to be characterized by a concentration-dependent character. These observations imply that AF is a promising agent that might be repurposed for the treatment of severe bacterial infections due to resistant Staphylococci; further investigations in animal models are needed. On the other hand, studies will be deepened to understand the effective molecular mechanisms through which auranofin manifests its potent antibacterial properties and to elucidate the reasons for the large differences in sensitivity between *S. aureus* and *E. coli*.

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